The effect of protein deficiency on the development of chronic antigen–antibody complex disease in mice

M. C. REINHARDT,* MADELEINE DEVEY,† M. COLLINS,† B. GREGORY* & M. W. STEWARD† * *Department of Immunology and Animal House, Institute of Child Health, London, and* † *Immunology Unit, Department of Medical Microbiology, London School of Hygiene and Tropical Medicine, London*

SUMMARY

Mice genetically selected to produce antibodies of either high or low affinity to protein antigens injected in saline were fed either a normal protein diet or a protein-deficient diet and were given daily injections of HSA for up to 73 days to induce chronic antigen–antibody complex disease. In low-affinity mice fed the normal protein diet, this resulted in impairment of renal function, deposition of immunoglobulin, C3 and HSA in the glomeruli, high levels of circulating antigen–antibody complexes and death from apparent renal failure in 50% of the animals. High-affinity mice on either diet had no impairment of renal function, fewer deposits in the glomeruli, lower levels of circulating complexes and no deaths. Low-affinity mice fed the protein-deficient diet had less impairment of renal function and less glomerular deposition of complexes than did low-affinity mice fed the normal diet. In addition, none of these mice died from renal failure. These results demonstrate that the protein-deficient diet reduced the severity of the experimental chronic antigen–antibody complex disease in low-affinity mice but did not increase the susceptibility of high-affinity mice to the disease.

INTRODUCTION

The affinity of the antibody response to protein antigens injected in saline into mice is under genetic control (Katz & Steward, 1975; Steward & Petty, 1976; Kim & Siskind, 1978) by mechanisms independent from those controlling antibody levels (Katz & Steward, 1975; Steward & Petty, 1976). The genes governing affinity are not linked to the major histocompatibility locus (Steward, Reinhardt & Staines, 1979). Environmental changes such as diet also affect the antibody response. In mice genetically predetermined or selectively bred to produce high-affinity antibodies, a protein-deficient diet during the period of antigen administration results in the production of low-affinity antibody (Passwell, Steward & Soothill, 1974; Reinhardt & Steward, 1979). The reduced antibody affinity resulting from protein deficiency could be due to depressed macrophage clearance function (Coovadia & Soothill, 1976), to defective T cell function or to impaired T–B cell co-operation (reviewed by Chandra & Newberne, 1977). Low-affinity antibody is poor at immune elimination of antigen (Alpers, Steward & Soothill, 1972) and the production of low-affinity antibody in mice is related to the development of chronic antigen–antibody complex disease (Soothill & Steward, 1971; Steward, 1979; Devey & Steward, 1980). Daily antigen injections resulted in higher levels of circulating antigen–antibody complexes, greater impairment of renal

function, significantly more complex deposition in the glomeruli and more deaths in animals selectively bred to produce low-affinity antibody as compared to animals selected to produce high-affinity antibody (Steward, 1979; Devey & Steward, 1980). The present study was designed to investigate the role of protein deficiency, which is known to reduce antibody affinity, in the induction of chronic antigen–antibody complex disease in both high- and low-affinity TO mice.

MATERIALS AND METHODS

Mice. Random bred TO mice were initially obtained from the Scientific Animal Service, Elstree, Hertfordshire, and subsequently maintained in the Animal House of the London School of Hygiene and Tropical Medicine.

Selection and breeding. Breeding was carried out from TO mice on the basis of the relative affinity (K_R) of antibody produced to the protein antigens human serum albumin (HSA) or transferrin injected in saline as described by Katz & Steward (1975). Mice producing either high- ($K_R > 10^6$ l/mol) or low-affinity antibody (K_R 10^4 to 10^5 l/mol) were selectively mated at each generation to give two lines referred to as the 'high-affinity' and 'low-affinity' lines respectively. All mice studied were of generation 12.

Dietary manipulation. Forty-eight high-affinity mice and 48 low-affinity mice aged 8–12 weeks were subdivided into two groups receiving a normal or low protein diet. Diets were made up as described previously (Reinhardt & Steward, 1979) and are expressed as weight of protein/weight of total dry diet (%). These values are very close to protein calorie/total calorie (%) as expressed by Coovadia & Soothill (1976). Mice were housed in the Animal House of the Institute of Child Health in metabolic cages and pair-fed so that mice on protein-deficient diets (4% protein) received the same amount of food as that eaten the previous day by mice on normal diets (15% protein). Wasted food was measured daily but was negligible. Animals were put on their diet 15 days before the first injection (day 1 of the experiment).

Injections. The 12 age- and sex-matched mice in each of four groups were injected daily with 0·25 mg HSA in 0·1 ml saline subcutaneously, the dose which produces the highest levels of circulating complexes and the most severe renal impairment in low-affinity mice (Devey & Steward, 1980). Control high- and low-affinity mice receiving either the 15% or the 4% protein diet were not injected. After 73 injections of antigen, surviving mice were killed and blood was taken by intracardiac puncture 36 hr after the last injection of HSA. Sera were stored at $-70°C$ until tested for antibody levels, antibody affinity and circulating antigen–antibody complexes.

Assessment of renal function. The glomerular filtration rate was assessed weekly by the measurement of the clearance of ^{51}Cr-EDTA from the blood (Knight, Adams & Purves, 1977). Each mouse received 2 μCi ^{51}Cr-EDTA intraperitoneally and an estimate of the blood half-life was obtained from samples taken 20 and 40 min after the injection, using the formula:

$$T_{\frac{1}{2}} = \frac{\ln 2\,(t_2 - t_1)}{\ln N_1 - \ln N_2}$$

where $t_2 - t_1$ is the time interval between blood samples and N_1 and N_2 are the c.p.m. in the blood samples.

Results were expressed as the ratio of the $T_{\frac{1}{2}}$ for each experimental animal (x) to a mean of the control $T_{\frac{1}{2}}$ (x/c).

Antibody assay. The amount (Ab_t) and relative affinity (K_R) of free antibody to HSA in sera were measured by an ammonium sulphate precipitation method to separate bound and free antigen, incorporating ^{22}Na as a volume indicator (Gaze, West & Steward, 1973).

Measurement of antigen–antibody complexes. Circulating antigen–antibody complexes were measured using a conglutinin-binding assay (KBA) and a C1q-binding assay (C1qBA) (Devey, Taylor & Steward, 1980). Briefly, polystyrene LP3 tubes (Luckham Ltd) were coated with C1q or conglutinin. For the C1qBA, sera were preincubated with EDTA and duplicate serum samples were diluted in phosphate-buffered saline containing 0·05% Tween 20. For the KBA, duplicate serum samples were diluted in veronal-buffered saline containing $1·5 \times 10^{-4}$ M Ca^{2+}, 5×10^{-4} M Mg^{2+} and

0·05% Tween 20. After incubation and washing, complex-like material was estimated from the binding of affinity-purified ^{125}I-labelled anti-mouse immunoglobulin antibody to the tubes.

Immunofluorescence. Kidneys were snap-frozen in *n*-hexane cooled in alcohol/dry ice. Six-micron cryostat sections were cut and stained directly with FITC-conjugated antisera to either mouse immunoglobulins, mouse $\beta1C/\beta1A$ or HSA (Nordic). The sections were examined under a Zeiss fluorescence photomicroscope by epi-illumination and the intensity of fluorescence was scored on a 0 to 3+ scale on coded kidney samples. Patterns of localization were also determined and photographs taken on 400 ASA Ektachrome film (Kodak).

Statistics. The significance of differences between groups of mice was tested using Student's *t*-test for continuous variables and the χ^2 test for discrete variables.

RESULTS

Renal function in uninjected controls

Previous studies had established that the mean half-life ($T\frac{1}{2}$) of ^{51}Cr-EDTA in 215 normal mice was $10·27 \pm 1·9$ min (Devey, unpublished results). In the uninjected control mice fed the normal diet, the mean $T\frac{1}{2}$ did not differ significantly from this but in uninjected control mice fed the 4% protein diet there was a marked increase in the mean $T\frac{1}{2}$ of ^{51}Cr-EDTA during the period of protein deprivation (Fig. 1). This is in agreement with the finding of reduced glomerular filtration in chronic malnutrition in both humans (Alleyne, 1967) and experimental animals (Ichikawa *et al.*, 1980) and is likely to be due to multiple factors.

Renal function during chronic antigen administration

Renal function during chronic antigen administration in high- and low-affinity mice receiving the normal diet was very similar to that previously reported (Devey & Steward, 1980). The mean $T\frac{1}{2}$ values of ^{51}Cr-EDTA in the injected animals (expressed as a ratio of that in the appropriate uninjected control group) are shown in Fig. 2a & b. In high-affinity mice (Fig. 2b) these values did not differ significantly between mice on the 15% and the 4% diet or between these and the uninjected controls at any time. In low-affinity mice receiving the 15% diet (Fig. 2a), clearance of ^{51}Cr-EDTA was significantly decreased compared to both the uninjected controls and high-affinity mice on days
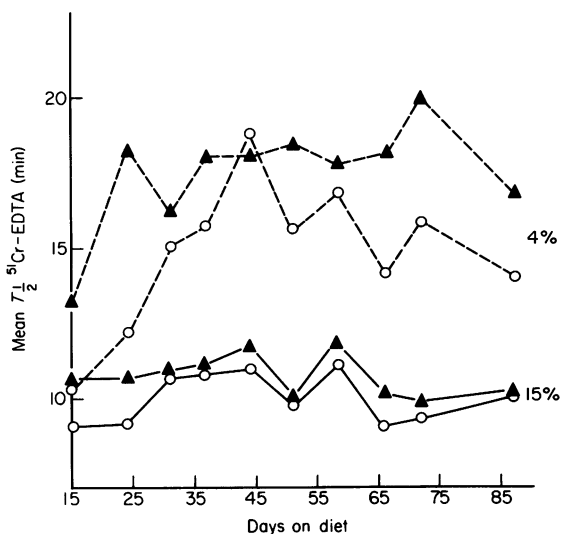

Fig. 1. Renal function in uninjected high-affinity mice (o) and low-affinity mice (▲) receiving the 15% diet (———) and the 4% diet (– – –). Points represent a mean of values from 12 mice.

22 ($P < 0.005$), 29 ($P < 0.05$), 36 ($P < 0.025$) and 51 ($P < 0.01$). By day 60 of the experiment, 50% of these mice had died from apparent renal failure. In contrast, injected low-affinity mice receiving the 4% diet (Fig. 2a) had significantly reduced clearance of ^{51}Cr-EDTA compared to the uninjected controls and high-affinity mice on day 29 only ($P < 0.01$). There was also a significant decrease in clearance in these mice compared to the high-affinity mice on day 43 ($P < 0.02$) but this value was not significantly different from that in the uninjected controls. There were no deaths from renal failure in low-affinity mice receiving the 4% protein diet.

Circulating antigen–antibody complexes

Circulating antigen–antibody complexes in serum samples taken from surviving mice on day 73 of the experiment (36 hr after the last injection of HSA) were measured by the solid-phase KBA and C1qBA (Fig. 3). In high-affinity mice on either diet, levels of circulating complexes did not differ significantly from those in the uninjected controls in either test. In low-affinity mice on the 15% diet, the levels of circulating complexes were significantly higher than those in uninjected controls by the KBA ($P < 0.02$) and the C1qBA ($P < 0.005$). In low-affinity mice on the 4% diet, complex levels were significantly higher than those in the uninjected controls by the C1qBA ($P < 0.001$) but not by the KBA ($P > 0.1$). There were significantly higher levels of complexes in injected low-affinity mice receiving the 15% diet compared to injected high-affinity mice receiving the 15% diet by both tests (KBA $P < 0.05$, C1qBA $P < 0.005$). In mice receiving the 4% diet, complex levels were significantly higher in injected low-affinity mice compared to injected high-affinity mice by the C1qBA only ($P < 0.005$). Despite the differences in complex levels between the high- and low-affinity lines described above, variation in the protein content of the diet did not itself cause a significant interline difference in the complex levels.

Immunofluorescence

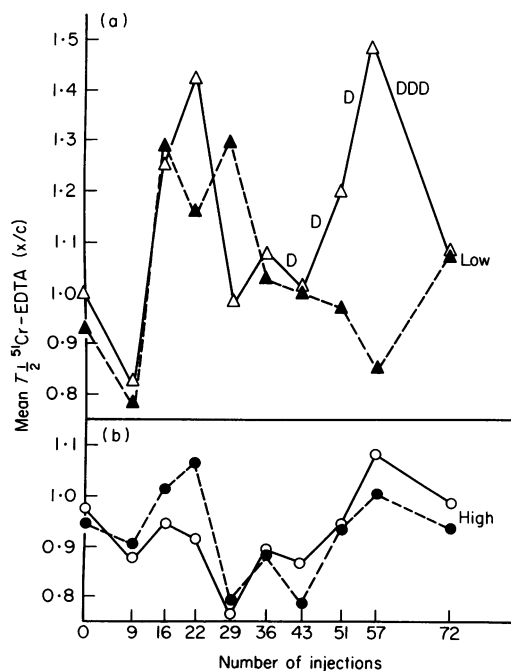Sections of kidneys, removed on day 73 of the experiment, were stained with FITC-conjugated

Fig. 2. Renal function in (a) low-affinity mice fed the normal diet (△—△) or the 4% diet (▲---▲) and in (b) high-affinity mice fed the normal diet (○——○) or the 4% diet (●- - -●) receiving daily injections of HSA. Points represent a mean of values from 12 mice. D = death.
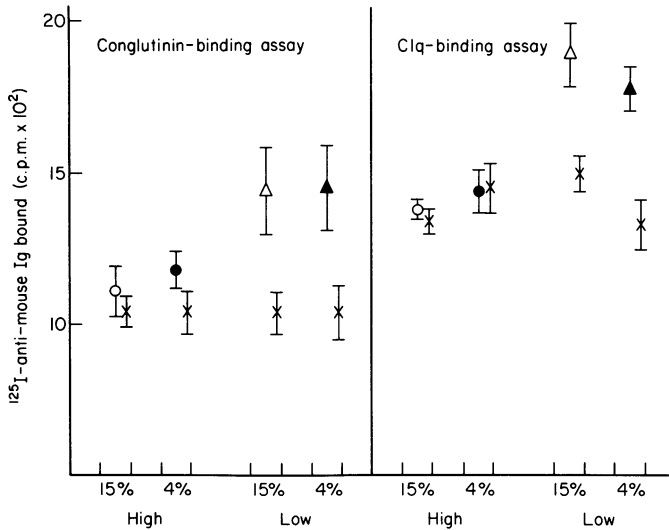
Fig. 3. Circulating antigen–antibody complexes measured by the conglutinin-binding assay and the C1q-binding assay in injected high (o) and low (△) affinity mice on the 15% diet and in injected high (●) and low (▲) affinity mice on the 4% diet. Points represent a mean of values from all the surviving mice at the end of the experiment (± s.e.). Values in uninjected control mice (×) are also shown (± s.e.).

antisera to mouse immunoglobulin (Ig), mouse C3 and HSA. The numbers of animals with positive fluorescence in their glomeruli are shown in Table 1. These results were analysed using a 2×2 contingency table and the χ^2 distribution. In high-affinity mice there was no significant difference between the incidence and intensity of fluorescence in the glomeruli of mice receiving the 15% diet compared to those receiving the 4% diet. In low-affinity mice, however, there was significantly more complex deposition in the glomeruli of mice receiving the 15% diet compared to those receiving the 4% diet for IgG ($P < 0.05$). In mice receiving the 15% diet, the incidence and intensity of fluorescence was significantly greater in low-affinity mice compared to high-affinity mice for IgG ($P < 0.01$), C3

Table 1. Incidence of fluorescence in the glomeruli of high- and low-affinity mice receiving daily injections of HSA

Line	Diet	Per cent mice showing positive glomerular staining with:		
		Ig	C3	HSA
High affinity	15%	30*	0*	10*
	4%	45	0	18
Low affinity	15%	80†	62	88
	4%	54	27	54

* Difference between high- and low-affinity lines on 15% diet significant for Ig ($\chi^2 = 9.1$, $P < 0.01$), C3 ($\chi^2 = 8.8$, $P < 0.01$) and HSA ($\chi^2 = 10.5$, $P < 0.01$).

† Difference between low-affinity mice on 15% and 4% diet significant for Ig ($\chi^2 = 4.9$, $P < 0.05$). The other differences were not significant due to the smaller sample sizes.

($P < 0.01$) and HSA ($P < 0.01$). However, in mice receiving the 4% protein diet, there was no significant difference in the amount of complex deposition between the two lines. Some fluorescence with FITC-anti-Ig was also seen in the glomeruli of uninjected control mice receiving the 4% diet and this may have been due to infection during the period of protein deprivation.

The localization of fluorescence in the glomeruli was also different between the two lines of mice on the different diets (Table 2). The predominantly glomerular basement membrane (GBM) localization of the fluorescent deposits (Fig. 4a) was seen only in low-affinity mice and although there was a greater incidence of this type of deposition in mice receiving the 15% diet compared to those on the 4% diet for Ig, C3 and HSA, the figures were not significant by χ^2 test. However, the presence of GBM localization was significant in low-affinity mice compared to high-affinity mice for Ig in mice on the 15% diet ($P < 0.05$), Ig in mice on the 4% diet ($P < 0.05$) and HSA in mice on the 15% diet ($P < 0.05$). The combined pattern of mesangial deposition of complexes with deposition in the contiguous GBM (Fig. 4b) was seen in both high- and low-affinity mice but predominantly in low-affinity mice receiving the 15% diet, while the deposition in the mesangium only (Fig. 4c) was seen only in high-affinity mice and some low-affinity mice receiving the 4% diet.

Characteristics of circulating antibody

The relative affinity (K_R) and total antibody levels (Ab_t) of free antibody remaining in the sera of surviving animals on day 73 are shown in Table 3. In high-affinity mice there was no significant difference in the mean Ab_t and K_R values between mice on the 15% and 4% diets. The Ab_t in low-affinity mice on the 15% diet was significantly higher than the Ab_t in low-affinity mice on the 4% diet ($P < 0.05$) and high-affinity mice on both diets ($P < 0.02$). Mean K_R was also significantly higher in low-affinity mice on the 15% diet compared to low-affinity mice on the 4% diet ($P < 0.02$), but in all the groups the K_R of the free antibody to HSA was low (below 5×10^5 l/mol).

Table 2. Localization of antigen–antibody complexes in the glomeruli of high- and low-affinity mice receiving daily injections of HSA

FITC-antiserum	Line	Diet	Mesangium	Mesangium +GBM	GBM
				Predominant pattern of localization*	
Mouse Ig	High affinity	15%	10	30	0†
		4%	18	27	0†
	Low affinity	15%	0	63	38
		4%	0	18	36
Mouse C3	High affinity	15%	0	0	0
		4%	0	0	0
	Low affinity	15%	0	38	25
		4%	0	9	18
HSA	High affinity	15%	0	10	0†
		4%	0	18	0
	Low affinity	15%	0	50	38
		4%	9	18	27

* Values represent percentage of animals showing positive fluorescence with the particular pattern of localization.

† Presence of GBM localization significant in low-affinity mice compared to high-affinity mice for Ig in mice on 15% diet ($\chi^2 = 4.7$, $P < 0.05$), Ig in mice on 4% diet ($\chi^2 = 4.9$, $P < 0.05$) and HSA in mice on 15% diet ($\chi^2 = 4.7$, $P < 0.05$).
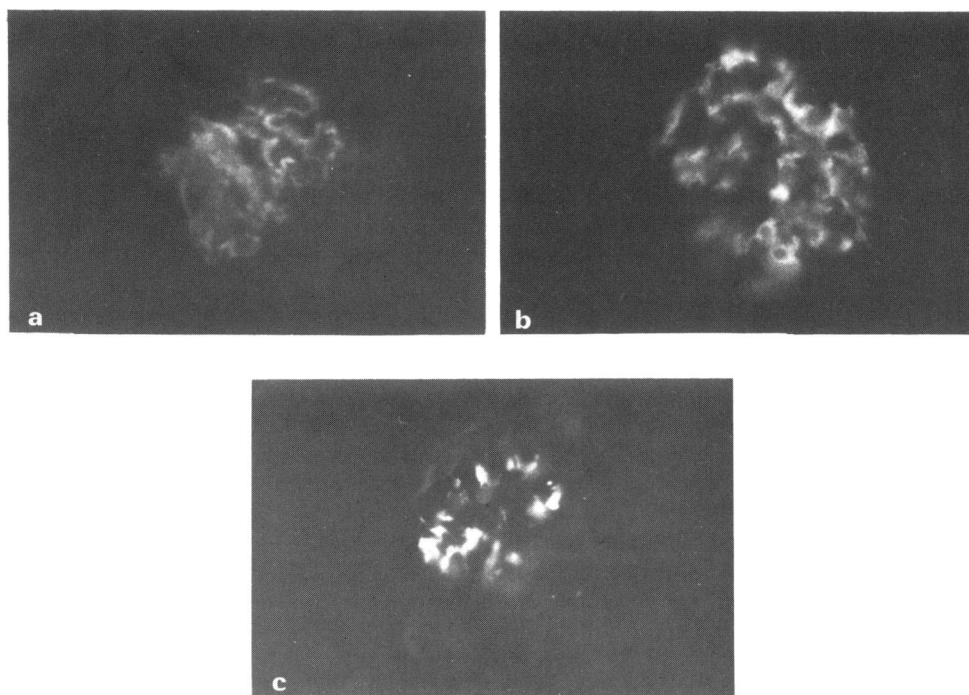
Fig. 4. (a) Predominantly GBM localization of Ig in a glomerulus of a low-affinity mouse receiving the 15% protein diet. (× 150.) (b) Combined mesangial and GBM localization of HSA in a glomerulus of a low-affinity mouse receiving the 15% protein diet. (× 150.) (c) Mesangial deposition of HSA in a glomerulus of a low-affinity mouse receiving the 4% protein diet. (× 150.)

Table 3. Levels (Ab_t) and relative affinity (K_R) of free anti-HSA antibody in sera after 73 injections of HSA

Line	Diet	Mean AB_t (pmol/10 μl serum)±s.e.	Mean K_R (× 10^6 l/mol) ±s.e.
High affinity	15%	363±86·6	0·34±0·08
	4%	227±45·5	0·27±0·03
Low affinity	15%	924±235·6†	0·40±0·09*
	4%	400±95·2	0·21±0·03

* Mean K_R in low-affinity mice on the 15% diet significantly higher than in low-affinity mice on the 4% diet ($P < 0.02$).

† Mean Ab_t in low-affinity mice on the 15% diet significantly higher than in low-affinity mice on the 4% diet ($P < 0.05$) and high-affinity mice on the 15% and 4% diets ($P < 0.05$).

DISCUSSION

The observation that low-affinity mice fed a 15% protein diet were more susceptible to chronic antigen–antibody complex disease following daily antigen injection than similarly fed high-affinity mice confirms previous findings (Steward, 1979; Devey & Steward, 1980). In these mice renal

function, assessed by the clearance of ^{51}Cr-EDTA from the serum, was impaired after 2 weeks of daily antigen injections (acute phase) followed by recovery and then, after 5–7 weeks of injections, by a second period of impairment (chronic phase) resulting in the death of 50% of the animals. In the present study the disease was more severe than previously found, possibly due to the longer period of antigen administration. In addition to the greater mortality in the low-affinity mice on the 15% diet, these animals also had a greater incidence and intensity of complex deposition in the kidneys and higher levels of circulating complexes in sera compared to the high-affinity mice on the 15% diet. Protein deprivation in low-affinity mice reduced the severity of the disease, since there were no deaths, impairment of renal function only at the acute stage of the disease and reduced complex deposits in the kidneys.

Inbred mice fed a normal diet produce high-affinity antibodies to protein antigens injected in saline, but produce low-affinity antibodies when the antigen is administered during a period of low dietary protein intake (Passwell *et al.*, 1974). This observation has been extended to the line of TO mice selected for its ability to produce high-affinity antibodies (Reinhardt & Steward, 1979). However, the 4% protein diet, although presumably reducing the affinity of antibodies to the antigen, did not increase the susceptibility of the high-affinity line to chronic antigen–antibody complex disease. In these animals there were no deaths, no impairment of renal function, no increased deposition of complexes in the kidney and no increase in levels of circulating complexes compared to high-affinity mice receiving the 15% protein diet. This could have been due to a number of reasons. Perhaps the most simple explanation is that protein deprivation reduces antibody levels (Table 3) and consequently the formation of antigen–antibody complexes in the kidneys would be restricted. However, the amount of circulating complexes in sera measured by the two solid-phase assays was not significantly lowered in protein deprivation (Fig. 3), but since the overall characteristics (e.g. size, class, complement-fixing ability, etc.) of the complexes in the different groups of animals were not assessed, the significance of this observation is difficult to determine. Also it has been shown previously that Ab_t is not reduced in protein deficiency following four once-weekly injections of antigen (Reinhardt & Steward, 1979). This may not be of significance in this present experiment as the animals were injected daily. Another, perhaps less likely, explanation is that the selection of TO mice (Katz & Steward, 1975) for antibody affinity could have affected properties of the kidneys which influence susceptibility to antigen–antibody complex deposition. These properties could include alterations in the ultrastructure or function of the glomerular mesangium (reviewed by Michael *et al.*, 1980), haemodynamic alterations affecting antigen–antibody complex trapping (Herbert, Allhuser & Koethe, 1978) or changes in the rate of monocyte infiltration related to differences in T cell-mediated immunity (Hunsicker *et al.*, 1979; Bradfield & Cattell, 1977).

Similarly, the reduction in the severity of antigen–antibody complex disease in low-affinity mice fed the 4% protein diet could be due to a number of factors. There was a reduction in the amount of free antibody in the sera but no significant decrease in circulating complexes; it is possible, of course, that although complexes were present, they were not those which could cause immunopathological changes in the kidneys. In the present experiment, animals on the 15% protein diet, which was isocaloric to the 4% diet, were pair-fed. Therefore, the observed differences could not have been due to low calorie intake, a suggested factor in the reduction of circulating antigen–antibody complexes in mice susceptible to auto-immune diseases fed diets with restricted calories (Safai-Kutti *et al.*, 1980). Passive transfer experiments have shown that antigen–antibody complexes which contain antibody of high affinity are large and poorly soluble and are taken up by the mesangium whereas complexes containing antibody of low affinity which are smaller and more soluble are localized within the glomerular basement membrane (Koyama *et al.*, 1978; Germuth *et al.*, 1979). It is possible that protein deprivation affected the properties (class, subclass, complement-fixing ability, etc.) of the antibodies leading to the production of larger, poorly soluble complexes which would localize in the mesangium resulting in an innocuous lesion. There is some evidence that, even in mice producing a predominantly low K_R antibody response, it is the limited high K_R subpopulation that is deposited in antigen excess in the kidneys (Steward, 1979). The fact that protein deprivation did not increase the susceptibility of high- or low-affinity mice to the disease could have been due to a reduction of this high K_R component leading to reduced deposition in the kidneys.

It is unlikely that the differences observed between high- and low-affinity mice are due to differences in systemic mononuclear phagocytic function as postulated by Ford (1975), Hoffstein *et al.* (1979) and Morgan & Soothill (1975). We have shown previously that both lines have similar phagocytic properties as assessed by the clearance of polyvinylpyrrolidone, preformed heterologous antigen–antibody complexes and heat-aggregated human IgG (Reinhardt & Steward, 1979). In our model of isocaloric protein deficiency, renal damage induced by the protein-deficient diet *per se* may have resulted in altered glomerular capillary permeability and this could have been responsible for the difference in the susceptibility to the chronic disease. If the disease was due to *in situ* formation and deposition of complexes (Mauer *et al.*, 1973; Izui *et al.*, 1977; Ford & Kosatka, 1979), environmental changes such as diet could cause differences in antigen trapping by the mesangium as demonstrated in various animal models (Mauer *et al.*, 1972; Hoyer, Elema & Vernier, 1976; Smith & Immekus, 1978). Other factors could include changes in the production of vasoactive amines due to diet and resulting in a different pattern of complex deposition (Kniker & Cochrane, 1968; Makker, 1977; Couser *et al.*, 1978).

An interesting finding in this study was the low affinity of the free antibody to HSA in the sera of all the groups of mice. This may well have been due to the binding of all available high-affinity antibody to the injected antigen, followed by rapid clearance from the circulation. As a result, the mean affinity of the antibody population remaining in the serum would be low. The fact that the sera were obtained only 36 hr after the final antigen injection may also have contributed to the reduction in the affinity of the free antibody.

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